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Characterization of Monospecies Biofilm Formation by *Helicobacter pylori*

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As all bacteria studied to date, the gastric pathogen *Helicobacter pylori* has an alternate lifestyle as a biofilm. *H. pylori* forms biofilms on glass surfaces at the air-liquid interface in stationary or shaking batch cultures. By light microscopy, we have observed attachment of individual, spiral *H. pylori* to glass surfaces, followed by division to form microcolonies, merging of individual microcolonies, and growth in the third dimension. Scanning electron micrographs showed *H. pylori* arranged in a matrix on the glass with channels for nutrient flow, typical of other bacterial biofilms. To understand the importance of biofilms to the *H. pylori* life cycle, we tested the effect of mucin on biofilm formation. Our results showed that 10% mucin greatly increased the number of planktonic *H. pylori* while not affecting biofilm bacteria, resulting in a decline in percent adherence to the glass. This suggests that in the mucus-rich stomach, *H. pylori* planktonic growth is favored over biofilm formation. We also investigated the effect of specific mutations in several genes, including the quorum-sensing gene, *luxS*, and the *cagE* type IV secretion gene. Both of these mutants were found to form biofilms approximately twofold more efficiently than the wild type in both assays. These results indicate the relative importance of these genes to the production of biofilms by *H. pylori* and the selective enhancement of planktonic growth in the presence of gastric mucin.

Helicobacter pylori, the primary etiological agent of gastritis, colonizes both the surface of gastric epithelial cells and the mucus gel layer. However, several recent studies have also identified *H. pylori* in water distribution systems (18, 29, 34, 38), surface wells or groundwater (16), rivers (11), and wastewater systems (17, 25, 29, 30) in several countries. In addition, *H. pylori* has been found existing on the inner surface of water storage earthenware in Gambia (3). While the mechanism of transmission of *H. pylori* is thought to be primarily by the fecal-oral route, the possibility that it may exist as a biofilm (most likely mixed species) on surfaces exposed to water may provide another route of infection.

Biofilms have been intensively studied as communities of bacteria able to survive in environments unfavorable to planktonic (free-living) bacteria (7, 24). In response to quorum-sensing signals, bacteria migrate and adhere to a surface, divide to form microcolonies, and expand laterally and vertically (8, 40, 48). Channels are formed in the biofilm to allow nutrients to reach bottom layers of bacteria. The mature biofilm is more resistant to killing by biocides and antibiotics, presumably due to the differentiation and survival of persister cells or slow growth rate (42). To further expand, sections of the biofilm shear off for colonization elsewhere (6).

Two studies have alluded to the ability of *H. pylori* to form a biofilm. A polysaccharide-containing biofilm has been observed at the air-liquid interface when *H. pylori* is grown in a glass fermenter (43). *H. pylori* is also capable of binding to a heterotrophic mixed species biofilm grown on stainless steel coupons (26). However, beyond determination of the sugars in the polymeric matrix, no further characterization of *H. pylori* biofilms has been done.

In the stomach, *H. pylori* exists primarily as swimming, planktonic bacteria, with a minority of bacteria adherent to the epithelium. Because mucin prevents *H. pylori* from binding to epithelial cells (12, 46) and prebound bacteria can be detached by washing with mucin (41), we were interested in the effect of mucin on *H. pylori* biofilm formation. We found that increasing concentrations of mucin significantly enhanced planktonic growth over biofilm formation.

H. pylori has a type IV secretion system (the *cag* pathogenicity island), which is known to be important for induction of interleukin-8 (IL-8) by gastric epithelial cells, as well as the secretion of bacterial proteins into host cells. We tested the effect of a specific mutation of this secretion system (*cagE*), which prevents assembly of the secretory apparatus, on biofilm formation by *H. pylori*. In addition, quorum-signaling systems are noted for their role in establishing communication among bacteria in a biofilm. *H. pylori* contains one such system, *luxS*, which has been shown to play a role in biofilm formation in *Streptococcus mutans* (31). We tested the effect of a mutation in this gene in *H. pylori* in two quantitative systems. Mutations in either *cagE* or *luxS* affected biofilm formation by *H. pylori*. Mutations in genes known to affect biofilm formation in other bacteria (response regulator, Clp protease, polyphosphokinase, and fibrillar hemagglutinin) did not affect biofilm formation.

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MATERIALS AND METHODS

Growth and microscopy of *H. pylori*. Table 1 lists the *H. pylori* strains used in this study. *H. pylori* strain SD14 is a self-aggregating *cagA*$^+$ strain from a duo-

TABLE 1. *H. pylori* strains

Strain	Phenotype	Origin	Reference	Source
SD3	Cag^+	Duodenal ulcer	5	San Diego
SD4	Cag^+	Duodenal ulcer	4	San Diego
SD7	Cag^+	Duodenal ulcer	This study	San Diego
SD14	Cag^+	Duodenal ulcer	4	San Diego
SD14*ppk*	Ppk^-	SD14	This study	San Diego
SD14*luxS*	$LuxS^-$	SD14	This study	San Diego
SD14*clpA*	$ClpA^-$	SD14	This study	San Diego
SD14*hpaA*	$HpaA^-$	SD14	This study	San Diego
SD14*HP1365*	Response $regulator^-$	SD14	This study	San Diego
SPM314[a]	Cag^-	Mouse adapted	27	R. Rappuoli
SPM326[a]	Cag^+	Mouse adapted	27	R. Rappuoli
SS1	Cag^{+b}	Mouse adapted	23	A. Lee
43504	Cag^+	Human antrum	28	ATCC[c]
26695	Cag^+	Gastritis	45	ATCC[c]
N6		Piglet adapted	10	K. Eaton
N6*flaA*	$FlaA^-$	N6	10	K. Eaton
N6*flaB*	$FlaB^-$	N6	10	K. Eaton
N6*flaAB*	$FlaA,B^-$	N6	10	K. Eaton
UA948*fucT*	Le^{x-d}	UA948	37	D. Taylor

[a] Strains not isogenic.
[b] Although *cag* pathogenicity island genes are present in this strain, it is unable to induce IL-8 in human gastric epithelial cells (47).
[c] American Type Culture Collection, Manassas, Va.
[d] Deficient in type I and II Lewis (Le) antigens (37).

denal ulcer patient and was described previously (4, 5). The aggregates produced by strain SD14 grown in broth are visible to the naked eye. Strains SD3, SD4, and SD7 were similarly isolated from duodenal ulcer patients; however, these strains do not appreciably self-aggregate in liquid culture.

Bacteria were grown from frozen stocks on Columbia agar plates containing 5% sheep or horse blood and 1% Fungizone (amphotericin B; Omega Scientific) in an atmosphere of 10% CO_2, 5% O_2, and 85% N_2. Bacteria were harvested at 2 to 3 days by scraping into brain heart infusion (BHI) broth containing 0.1% β-cyclodextrin (CD; Sigma) or 5% heat-inactivated (56°C, 20 min) fetal calf serum (ΔFCS). Because strain SD14 forms aggregates in broth culture, bacterial clumps of this strain were fragmented by multiple passages through a 25-gauge needle, and single bacteria were enumerated using a Petroff-Hauser counter. Our investigators have found previously that passage through this size needle did not alter the ability of the organism to adhere to gastric epithelial cells or to induce IL-8 production in these cells (4, 5). Cultures were adjusted to 5×10^7 cells ml^{-1}, and 1 to 2 ml was inoculated per well into six-well microtiter trays. Each well also contained a sterile 25-mm borosilicate coverslip that had been placed at an angle in the chamber in order to allow biofilm formation at the air-liquid interface, and the cultures were gently shaken on an orbital shaker for 2 to 6 days. Alternatively, stationary bacteria were grown in eight-chamber slides (300 μl/well; Lab-TekII; Nalge Nunc) with 12-mm borosilicate circular coverslips inserted perpendicular to the slide surface.

Coverslips containing *H. pylori* biofilms were fixed for 1 to 2 h in 5% formalin and stained with carbol-fuchsin for light microscopy. Unfixed coverslips were also stained with the Live/Dead BacLight kit (Molecular Probes) as described by the manufacturer. Samples on glass slides were prepared for scanning electron microscopy (SEM) by fixation in 5% formalin and successive dehydrations through a graded series of alcohol, followed by coating with gold-palladium. SEM micrographs were obtained from the Electron Microscopy Core Facility at San Diego State University on a Hitachi S2700 instrument.

Quantitation of biofilm formation. Because *H. pylori* grows slowly, becomes nonculturable but viable with extended culture, does not adhere appreciably to plastic, and only forms a biofilm at the air-liquid interface, new methods were devised for quantitation of biofilm formation.

In the first method, individual respiratory activities of planktonic and biofilm bacteria grown in the same microtiter well were compared using a method adapted from quantification of biofilms of *Candida dubliniensis* (35). Wells in a 12-unit microtiter tray were filled approximately half full with autoclaved coarse glass frit (approximately 2- to 5-mm diameter; Bullseye Glass) to allow adherence of *H. pylori* at the air-liquid interface. *H. pylori* strains were grown for 2 days on agar, dispersed into BHI broth containing 0.1% CD (for the mucin experiments) or 5% ΔFCS (for mutant experiments), counted, and adjusted to 5×10^7 cells ml^{-1}. For experiments with mucin, BHI broth was autoclaved together with type II (crude) mucin from porcine stomach (Sigma) at the percentages indicated below in Fig. 4, and 0.1% CD was added prior to incubation. One milliliter of bacteria was inoculated per well in 12-well microtiter trays containing the frit. Preliminary experiments indicated that maximal biofilm formation occurred at 5 days with CD and at 3 to 4 days with ΔFCS; therefore, cultures were incubated at 37°C for 3 to 5 days as appropriate for the additive, with or without gentle shaking (as indicated in the figure legends) in the same atmosphere as described above. For quantitation, planktonic bacteria were removed from the mixture and assayed separately from the remaining biofilm bacteria using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma). In preliminary experiments, we found that incubation of *H. pylori* with MTT gave higher values and less background absorbance than other tetrazolium reagents in BHI broth (data not shown). We also found that coccoid *H. pylori* cells (obtained from 7- to 10-day-old agar plates) isolated on a 20-to-80% Percoll (Pharmacia) step gradient were approximately 40% as metabolically active as spiral forms isolated from the same gradient (data not shown). MTT is reduced during respiration to form the water-insoluble, purple formazan, which may be extracted from the bacteria with isopropanol for absorbance measurements. Nonadherent, planktonic bacteria were aspirated from the remaining biofilm bacteria colonizing the glass frit, and both populations were assayed separately, as follows. (i) For biofilms, the glass frit containing adherent *H. pylori* cells was washed twice with phosphate-buffered saline (PBS). A 1.5-ml aliquot of sterile MTT (0.5 mg ml^{-1}) in BHI broth containing 0.1% CD was added to the glass frit, and the biofilms were incubated with gentle shaking in the same atmosphere as described above for 24 h. This amount of time was required since the growth rate of *H. pylori* is slow under these conditions ($t_{1/2} > 12$ h). The solution was aspirated from the frit containing the adherent, purple-stained bacteria, and 1.5 ml of isopropanol was added to the frit to extract the formazan. (ii) For planktonic bacteria, nonadherent *H. pylori* cells, including bacteria from the first wash of the glass frit with PBS, were pelleted ($15{,}000 \times g$; 5 min), resuspended in 1 ml of MTT in broth as above, and incubated with shaking in a separate 12-well tray for 24 h. Planktonic bacteria were pelleted ($15{,}000 \times g$; 5 min) and resuspended in 1 ml of isopropanol to extract the formazan for absorbance measurements.

The isopropanol suspensions from both biofilm and planktonic cultures were pelleted ($15{,}000 \times g$; 5 min), supernatants were removed, and the absorbance of the supernatants at 550 nm was measured in an Ultrospec III spectrophotomer (Pharmacia LKB). Absorbance values were adjusted to reflect the differences in volumes of isopropanol-extracted planktonic and biofilm bacteria (1 ml versus 1.5 ml, respectively). Percent adherence was calculated as the (adjusted) A_{550} for the biofilm bacteria divided by the sum of the planktonic plus (adjusted) biofilm absorbances (35). All quantitative experiments were performed in quadruplicate, with at least two repetitions of the experiment.

The second method for quantitation involved direct counting of both planktonic and biofilm bacteria grown in the same well. For this, 1 ml of bacteria was inoculated at 5×10^7 ml^{-1} into six-well microtiter trays filled approximately half

TABLE 2. Primers and enzyme restriction sites used to construct *H. pylori* mutations by insertion of the modified *cat* gene

Gene	*H. pylori* 26695 gene[a]	Restriction site (bp)[b]	Sequence (5′-3′)[c]
clpA	HP0033	StyI (32851)	F: GGACAAAGCCAAAGCGTTAG
			R: CGGTATTCTTCAAAAGTGTCG
ppk	HP1010	NsiI (1073285)	F: CCAAAAAATCCAAGCCCTAC
			R: CGCTAAAAAAACTCACATCGG
hpaA	HP0410	NheI (424060)	F: CATTACCTCTAAACCACCCAGGAACG
			R: CAATCTGATGCTCTAACCGACTGAG
Response regulator	HP1365	None; 141-bp deletion[d]	F: GCATCAACACAAACGAAGAGCC
			R: ACCGCCTTACAGGATAACGCTAC
			F: CGCTCAACAGAGAGCCTTTCATAA
			R: TGAATTGCACCACATTGTGCC

[a] Gene numbers from the *H. pylori* 26695 sequence used to construct the mutations (45).
[b] Restriction sites used to insert the *cat* gene. Numbers indicate the base pair number cited in the *H. pylori* 26695 sequence (45).
[c] Primers used for PCR with genes from *H. pylori* strain 26695 for mutation and transformation into *H. pylori.* F, forward primer; R, reverse primer.
[d] For mutation of response regulator HP1365, the two primer sets shown were used for PCR with 5′ end and 3′ end, respectively. The modified *cat* gene was inserted in the opposite orientation between the two PCR products.

full with autoclaved glass frit. Bacteria were grown in BHI broth containing 0.1% CD, and the bacteria were grown with shaking for 5 days. Planktonic bacteria were aspirated, fixed with 2% formalin, and counted under polarized light using a Petroff-Hauser counter. Four squares were counted from replicates of four wells, and results were averaged. Biofilm bacteria remaining on the glass frit were washed twice with PBS, resuspended in PBS with 2% formalin, and sonicated for 3 min in a water bath. Bacteria released from the frit were aspirated and counted as above, and the percentage of adherent bacteria was calculated as for the respiratory assay.

Construction of the *luxS* mutant in *H. pylori*. Two *Escherichia coli* clones were obtained from the American Type Culture Collection (GHPDN83 and GHPEF23) containing segments of the genome of *H. pylori* strain 26695 surrounding the *luxS* gene (gene fragment HP0104, nucleotides 112673 to 110931; fragment HP0106, nucleotides 114475 to 113336; both minus strand [45]). The chloramphenicol resistance (*cat*) gene of *Campylobacter jejuni* (kindly provided by H. Mobley, originally from Diane Taylor) was engineered to remove the transcriptional stop site and ligated in the same orientation between the two clones. Primers used for the PCR with the *cat* gene were 5′-CGGGATCCTCG GCGGTGTTCCTTTCCAAG-3′ (forward primer) and 5′-GCTCTAGACTCG AGCGCCCTTTAGTTCCTAAAGGGT-3′ (reverse primer). Insertion of the *cat* gene resulted in a deletion of 162 bp starting 15 bp downstream of the *luxS* start site. Because of the removal of the transcriptional stop site in the *cat* gene, this deletion was not expected to disrupt the *metB* or *cpdB* genes located upstream or downstream, respectively, of the *luxS* gene. In addition, a construct with the opposite orientation of the *cat* gene with respect to the *luxS* coding sequence was made. Plasmid DNA was prepared from *E. coli* DH5α, and 5 to 10 μg was used to transform *H. pylori* strains SD14, SD3, and SS1 on nonselective agar plates, as described previously (13). Following overnight incubation, the bacteria were scraped from the surface and inoculated onto plates containing 20 μg of chloramphenicol ml^{-1}. Resistant colonies were subjected to Southern hybridization to confirm the disruption of the *luxS* gene. Also, oligomers were designed to a region upstream of the *luxS* gene and to the *cat* gene for PCR confirmation of the mutation in *H. pylori*.

In order to confirm the functional disruption of the *luxS* gene, the *Vibrio harveyii luxN* reporter strain BB170 (capable of sensing the LuxS autoinducer; kindly obtained from Bonnie Bassler) was used. The bioassay was performed as described previously (21), with the exception that the microtiter wells containing BB170 and *H. pylori* SD14 derivative supernatants were exposed to X-ray film and spots were quantified by densitometry. Essentially, SD14 and SD14*luxS* were grown in broth to a cell density of 6×10^8 ml^{-1}, and an equal amount of cell-free culture supernatant was mixed with a stationary-phase culture of *V. harveyii* BB170 in a microtiter dish.

Construction of additional mutants of *H. pylori*. The *cagE* mutant of *H. pylori* SD4 was previously described (15). The *ppk, clpA, hpaA*, and response regulator HP1365 mutations were constructed by PCR of the genes from *H. pylori* 26695, cloning into PCR-scriptSK (Stratagene), insertion of the modified *cat* gene, and transformation in *H. pylori* as described for the *luxS* mutant. Table 2 shows the gene identification numbers and the primers used to clone the genes, as well as the restriction sites used to insert the *cat* sequence for gene disruption. We were unable to obtain transformants of the *clpB* gene knockout in *H. pylori* SD14 with the *cat* gene in either orientation, suggesting that the mutation was lethal in this strain. Since this mutation was originally reported in strain N6, we attempted to transform *H. pylori* N6 with the inactivated *clpB* gene construct, made in *E. coli* and kindly provided by Elaine Allan (1). However, no mutants resulted from this transformation. In addition, mutation in the large *clpP* subunit gene was also lethal after a few passages on agar. We also tried to transform *H. pylori* SD14 with a mutated version of the *folP* gene, which is involved in anaerobic metabolism, but no mutants were obtained, suggesting that the gene is absolutely required for growth.

RESULTS

Growth as a biofilm. Figure 1 shows the progression of a typical biofilm of *H. pylori* SD14 at 3 to 4 days postinoculation. The bacteria adhered to the glass surface individually at the air-liquid interface and then divided to form circular microcolonies. The microcolonies grew to eventually merge together, forming a solid zone of bacteria at the air-liquid interface by 3 to 5 days, with or without gentle agitation. The three-dimensional architecture typical of other biofilms was observed for *H. pylori* in the SEM micrograph shown in Fig. 2. The bacteria (primarily coccoid in this micrograph) were stacked several layers thick, and numerous holes or pores could be seen extending to the base of the biofilm. As shown in the SEM micrograph, coccoid bacteria remained firmly attached. No obvious slime layer could be detected by SEM, but this could be due to the fixation of bacteria prior to processing for SEM (35). In our hands, *H. pylori* strains did not routinely form biofilms on plastic surfaces, such as the polystyrene used in microtiter trays.

The cells of the actively growing biofilm (3 to 4 days old) were alive, as all of the bacteria fluoresced green (excluded propidium iodide) when exposed to the Live/Dead BacLight kit. At 5 days, more extensively colonized sections of the biofilm could be seen starting to peel off in sheets, and some dead bacteria could be seen (still adherent to the biofilm). Coccoid bacteria could be seen in the older biofilms, but most of these bacteria continued to fluoresce green with the BacLight stains.

Biofilm formation by multiple *H. pylori* strains. All of the wild-type strains shown in Table 1 were able to form biofilms. This includes a gnotobiotic piglet-adapted strain (N6), mouse-adapted strains (SS1, SPM314, and SPM326), clinical isolates (SD3, SD4, SD7, and SD14), and laboratory strains (ATCC 26695 and 43504). The biofilms produced by all of these strains

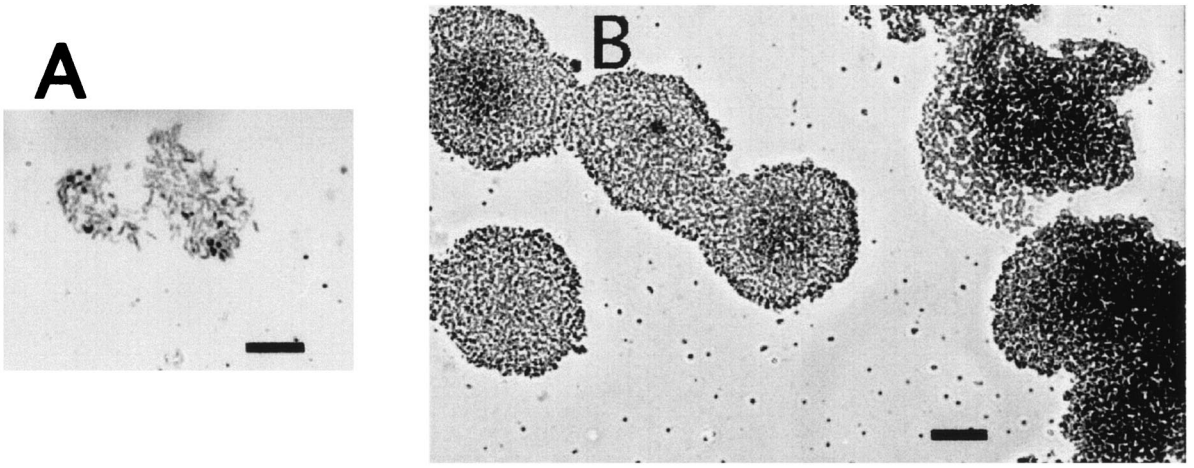

FIG. 1. Progression of biofilm formation by *H. pylori* SD14, as shown in light micrographs of fixed and carbol fuchsin-stained *H. pylori* SD14 cells at the air-liquid interface on borosilicate glass coverslips. (A) Single bacteria attached to glass at 3 days of stationary growth in broth. Attached bacteria began dividing in two dimensions to form a microcolony at 3 to 4 days. Bar, 10 μm. (B) Individual microcolonies merged into a dense biofilm at the air-liquid interface at >4 days of growth. Bar, 25 μm.

had similar three-dimensional architecture, observed by microscopy.

Effect of mucin on biofilm formation. Mucin prevents *H. pylori* from binding to epithelial cells (12, 41, 46); therefore, we investigated its effect on adherence of *H. pylori* to a glass surface. As shown in Fig. 3, mucin significantly increased planktonic growth in a dose-dependent manner, while the numbers of biofilm bacteria were not appreciably altered, leading to a substantial decrease in the percent adherence. This was true using both the respiratory assay with strain N6 (Fig. 3A and B) and direct counts with strain SD4 (Fig. 3C and D).

Effect of the *luxS* mutation on biofilm formation and coaggregation. The *luxS* gene is the only known quorum-sensing gene present in the sequenced *H. pylori* genome. The *luxS* genes of *H. pylori* (21) strains SD14, SS1, and SD3 were disrupted by allelic exchange using the *cat* gene. The gene disruption was confirmed by both genetic analysis and bioassay. Southern blotting assays were performed using both the *cat* gene and the *luxS* gene on genomic DNA from strain SD14 and the isogenic *luxS* mutant. The blots showed an appropriate-sized band in the *luxS* mutant (data not shown), consistent with a *cat* cassette inserted in the *luxS* gene. The mutant was also confirmed by PCR using oligomers specific for the *cat* gene (3′) and a region upstream from the *luxS* gene (5′). The resulting fragment corresponded in size to the 5′ end of the *luxS* gene, suggesting that the *cat* cassette had indeed been inserted in the *luxS* gene. For confirmation by bioassay, we examined the ability of the *H. pylori* strains to stimulate the *V. harveyii* reporter strain BB170 to luminesce. The relative densities or light units for strain BB170 mixed with medium alone, *H. pylori* SD14, or *H. pylori* SD14*luxS* were 0.74, 1.17, and 0.73, respectively. Although the light output of the wild-type strain was less than those of rapid-growth bacteria such as salmonellae, the *H. pylori luxS* mutant induced light formation by *V. harveyii* less than wild type, confirming the mutation.

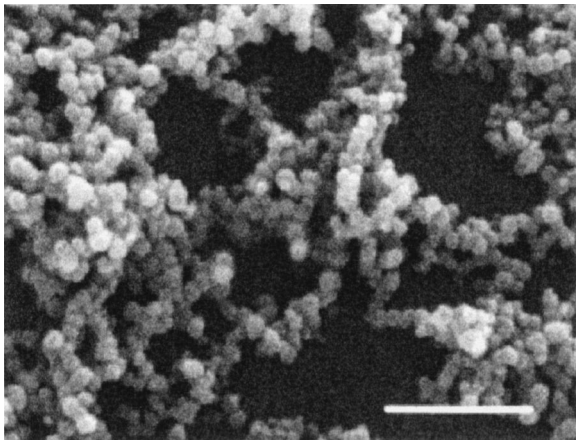

FIG. 2. SEM micrograph of a 4-day-old biofilm of *H. pylori* SD14 grown as a stationary batch culture. Bar, 1 μm.

Although the *luxS* mutant had a similar growth rate to the wild type, we found that the mutant in both strains tested, with *cat* in either orientation, was capable of forming a mature biofilm microscopically similar to its isogenic parent. The bacteria were able to adhere, form microcolonies, and develop a three-dimensional architecture identical to the wild type. As planktonic bacteria, the mutant grew equally well in broth, self-aggregated (strain SD14*luxS*), and older cultures became coccoid, similarly to the wild type. In order to quantitate the amount of biofilm formed by the *luxS* mutant, it was compared to the wild type in the two assays described in Materials and Methods. As shown in Fig. 4A, the *luxS* mutant of strain SD3 was over threefold better at forming a biofilm than the wild-type parent strain in the respiratory assay. In addition, the same mutation in strain SD14 produced approximately twofold more biofilm than the wild type in the direct count assay, as indicated in Fig. 4B. We conclude that the lack of quorum sensing actually increased biofilm formation in two *H. pylori* clinical strains.

Effect of the *cag* type IV secretion system on biofilm formation. Inactivation of the *cagE* gene results in the inability to assemble the type IV secretion apparatus (reviewed in refer-

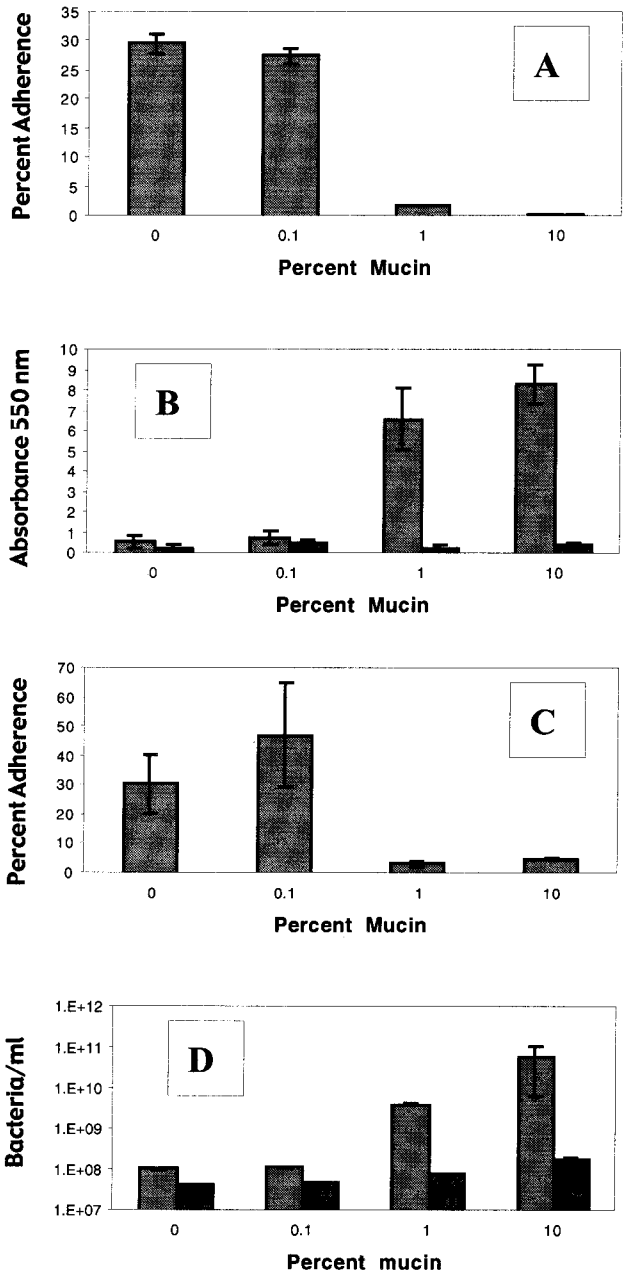

FIG. 3. Effect of mucin on biofilm production. Biofilm formation of *H. pylori* strains in the presence of 0 to 10% mucin was compared using the MTT respiratory assay (strain N6) (A and B) and direct counts (strain SD4) (C and D). Percent adherence (A and C) was calculated as described in the text for the two assays. In panels B and D, dark bars represent quantitation of biofilm bacteria and light bars indicate absolute values for planktonic bacteria. In both assays, note that while the percent adherence decreased with 1 and 10% mucin, the planktonic bacteria greatly outnumbered the biofilm bacteria (which remain relatively unchanged in number) in both assays. Error bars represent means ± standard deviations.

ence 39). We tested the effect of the *cagE* mutation on biofilm formation using the two assays described for the *luxS* mutant. Four separate isolates of the *cagE* mutant in strain SD4 were tested in the respiratory assay for their ability to form biofilms. As shown in Fig. 5A, all four isolates formed approximately twofold more biofilm than the wild-type strain. In addition, the *cagE* mutant consistently produced higher percentages of biofilm than wild-type SD4 in the direct count assay. This was confirmed in four independent experiments, with assays performed on 3-, 4-, and 5-day-old biofilms. In all cases, the *cagE* mutant produced between two- and fourfold more biofilm than the wild-type strain. A typical experiment is shown in Fig. 5B, which indicates that the *cagE* mutant of SD4 adhered to glass 2.8-fold more than the wild type in biofilms grown for either 3 or 5 days.

Effect of other mutations on biofilm formation. The *clpA, ppk, hpaA*, and response regulator HP1365 mutations were made in *H. pylori* SD14 and confirmed by PCR using primers outside of the inserted gene segments and within the *cat* cassette. All mutants were able to adhere to glass coverslips, and the resulting biofilms appeared similar to the wild type microscopically.

We tested the ability of a defined mutant deficient in terminal fucosylation of Lewis antigens (UA948 *fucT*) (37) to form a biofilm in our system. In *H. pylori*, the α(1,3/4)-fucosyltransferase is required for fucosylation of both type I (Le^a) and type II (Le^x) Lewis antigens; thus, mutation in the *fucT* gene eliminates all terminal fucosylated carbohydrates on the lipooligosaccharide (37). This mutant was capable of forming a dense biofilm at the air-liquid interface on glass coverslips, as observed microscopically.

Because none of these mutations showed significant differences in biofilm formation on glass coverslips, we did not submit them for further testing using the two quantitative assays.

DISCUSSION

The progression of biofilm formation by *H. pylori* mimics other biofilms described in the literature, beginning with individual bacteria adhering to the abiotic surface, expansion into colonies, and formation of a three-dimensional structure (Fig. 1 and 2). All strains tested were able to form biofilms on glass with overall similar biofilm structure (i.e., multiple layers of bacteria with channels for nutrient flow), including clinical isolates and laboratory strains, as well as mouse-adapted strains. *H. pylori* formed a biofilm only at the air-liquid interface, which is most likely indicative of its microaerobic, capnophilic character.

A number of global regulatory genes, controlling inducible genes such as those involved in virulence, cell-cell signaling, and responses to environmental signals, have been implicated in biofilm formation (reviewed in references 6 to 8, 19, 22, and 40). We investigated the importance of several genes involved in global regulation on biofilm formation by observing specific mutants in *H. pylori*. Because *H. pylori* has a paucity of potential global regulators (45), we made specific mutations in genes known to affect biofilm formation in other systems. Although the polyphosphokinase (Ppk) is required for biofilm formation in other gram-negative bacteria (32, 36), this does not appear to be the case for *H. pylori*. Likewise, although Clp protease mutations affect biofilm formation in *Pseudomonas fluorescens* (33), mutation in the *clpA* small subunit did not inhibit biofilms by *H. pylori*. However, it is possible that other Clp small sub-

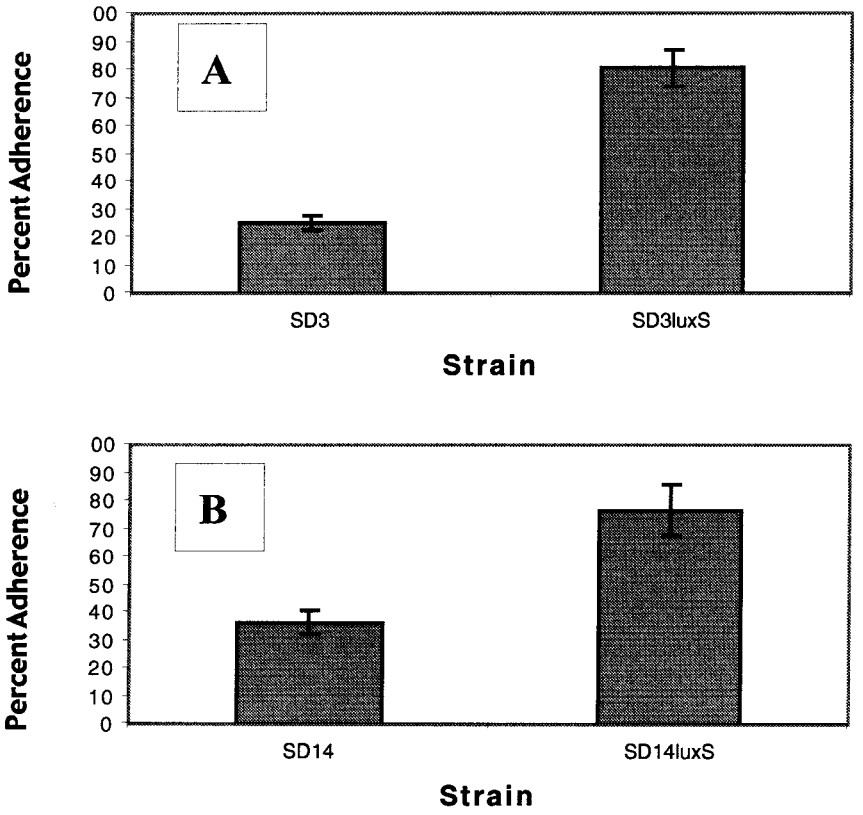

FIG. 4. Effect of the *luxS* mutation on biofilm formation by two different strains of *H. pylori*. (A) Three-day-old *H. pylori* SD3 and SD3*luxS* biofilms were quantitated using the respiratory assay, as described in the text. (B) SD14 and SD14*luxS* were grown in biofilms for 3 days and quantitated using the direct count assay, as described in Materials and Methods. Error bars represent means ± standard deviations.

units (ClpB or ClpX) affect this process. A number of studies have implicated two-component regulatory systems in controlling biofilm formation. The *H. pylori* 26695 genome has four homologous two-component sensor kinases and six genes encoding response regulators (45). Three of the response regulators are essential, and two are involved in flagellar and chemotaxis regulation (2). Therefore, we decided to inactivate the remaining response regulator, which exists at the beginning of an operon with its (presumably) cognate sensor (2). However, this mutant was able to form a biofilm essentially equivalent to that of the wild type, suggesting that the gene is not critical to biofilm formation. Again, this does not exclude the importance of other two-component systems in biofilms.

The quantitation of biofilm formation by *H. pylori* is complicated by a number of factors. For example, *H. pylori* only forms a biofilm at the air-liquid interface and most strains do not form biofilms on plastic, similarly to other bacteria reported. In addition, *H. pylori* requires extended incubation (≥3 days of growth) in a microaerobic atmosphere for biofilm formation and readily converts to the viable but nonculturable (VBNC) form with extended incubation. Therefore, care had to be taken to choose quantitative assays that addressed these issues. The respiratory assay was chosen, since the ability to reduce tetrazolium salts remains high in VBNC *H. pylori*, at least under certain growth conditions (14). We also found that coccoid *H. pylori* cells from 7- to 10-day-old cultures respire at 40% of the rate of spiral forms (data not shown), a level far greater than that reported for protein synthesis or ATP content in *H. pylori* VBNC forms. In addition, quantitation of both planktonic and biofilm bacteria using tetrazolium salt reduction has been previously described for other difficult-to-culture bacteria (9, 35). However, because biofilm bacteria may not respire at the same rate as planktonic bacteria, we developed a second assay using direct counts of both planktonic and biofilm *H. pylori*. Direct counts are the preferred method for quantitation over colony counts, since biofilm formation required 3 to 5 days of incubation, which can produce many VBNC bacteria.

Using both of these assays, we found that mucin greatly accelerated planktonic growth over expansion of biofilm *H. pylori*. In the stomach, mucus is thought to form a protective layer to infection, since it is continually swept out by muscular contractions. In fact, *H. pylori* is chemoattracted to and binds human gastric mucin, the major component of mucus (reviewed in reference 44). Mucin is known to increase the growth of *H. pylori* in broth cultures (20). Our data complement these findings for planktonic cultures of *H. pylori*, showing that planktonic growth is enhanced with mucin addition. However, we also found that while planktonic growth was enhanced, mucin did not inhibit biofilm formation or affect the number of adherent bacteria, as shown in Fig. 3. Therefore, for the first

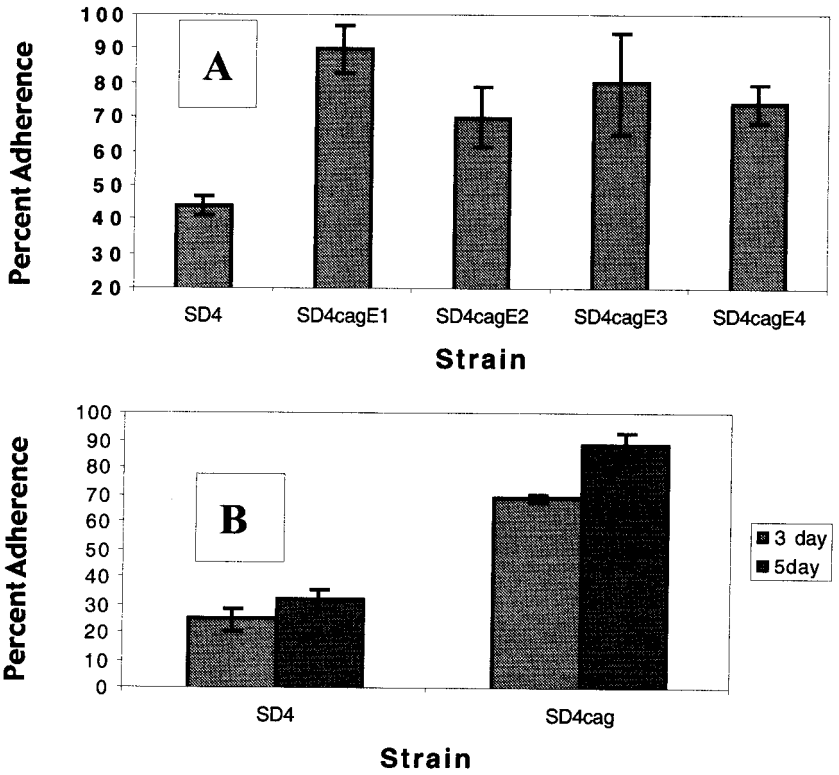

FIG. 5. Effect of the *cagE* mutation on biofilm formation. (A) *H. pylori* SD4 and four different *cagE* isolates were grown as biofilms for 3 days and quantitated using the respiratory assay, as described in the text. (B) Strains SD4 and SD4*cagE* were grown as biofilms and assayed by direct counts, as described in Materials and Methods. Light bars represent results from biofilms grown for 3 days, while dark bars indicate values for 5-day-old biofilms. Error bars represent means of four experiments ± standard deviations.

time, our studies show the effect of mucin on biofilms produced by bacteria normally found in the gastrointestinal tract. These results were corroborated by the use of two quantitative assays and two different strains (N6, a mouse-adapted strain, and SD4, a clinical isolate). As a growth promoter, mucin may be used as a carbon source or may serve to modify the viscosity of the solution, but the growth-enhancing benefits of this supplement only pertained to planktonic bacteria. Therefore, we suggest that as *H. pylori* encounters mucin in the stomach, the selective growth of planktonic bacteria greatly accelerates, which explains the large number of free-swimming bacteria observed in the mucus layer.

Recently, the *luxS* gene in *S. mutans* was shown to be involved in biofilm formation (49); therefore, we tested the effect of LuxS on biofilms produced by *H. pylori*. Interestingly, a mutation in *luxS* actually increased monospecies biofilm formation by *H. pylori* in multiple experimental trials and with two separate strains. The mechanism for this enhancement of biofilm formation is likely to be complex; nevertheless, this represents the first phenotype associated with the *luxS* mutation in *H. pylori*.

Equally surprising was the effect of the *cagE* mutation on *H. pylori* biofilm formation. The Cag type IV secretion system forms a large membrane pore and contains many protein components. While a few components have been found to be secreted by this system, it is likely that other products are secreted through this apparatus. We found that an isogenic mutant lacking a functional *cagE* gene reproducibly adhered more strongly to glass surfaces than the wild type. Since this occurred with multiple isolates from the allelic replacement transformation, it is unlikely that some untoward effect from the *cagE* gene disruption (for example, mutation at another site) was responsible for these results. The reason for these findings remains obscure. The inability to secrete certain components could pertain to these findings, or perturbation of the membrane surface could result in nonspecific effects, such as alteration of membrane pH or hydrophobicity. It is possible that, since the *cag* pathogenicity island may not be required, or even beneficial, for survival outside the host, the additional energy cost of transcribing this operon hinders efficient biofilm formation. The ability to form a biofilm may be a remnant of a more primitive (premammalian) lifestyle for *H. pylori*. In addition, our data suggest that there may be an interplay between the *luxS* and *cagE* genes, although investigation of these interactions remains beyond the scope of this paper. Whatever the explanation for the effect of the *cagE* mutation on biofilm formation, our findings suggest a novel phenotype for the Cag type IV secretion system.

As mentioned in the introduction, *H. pylori* has been identified in multiple water systems throughout the world. We believe that the adherence of *H. pylori* to glass (43), stainless steel (26), cast iron (34), and clay represents a novel habitat and may allow growth in the environment at the air-liquid

interface. For example, *H. pylori* may form biofilms on rocks containing silicates, iron, or other clay minerals at the air-liquid interface in surface wells. Our results indicate the relative importance of a number of genes to the production of biofilms by *H. pylori* and indicate the selective enhancement of planktonic growth in the presence of gastric mucin. We suggest that *H. pylori* exists primarily as a biofilm in the environment and, upon encountering gastric mucus, rapidly proliferates as planktonic bacteria in the stomach.

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